Skeletal Muscle Myosin Monomer in Equilibrium with Filaments Forms a Folded Conformation*

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Rabbit skeletal myosin forms stable filaments under physiological conditions, and only a small amount stays as a monomer in equilibrium with filaments. The myosin monomers were observed in two conformational states, as extended and folded forms upon electron microscopy and gel filtration high performance liquid chromatography. The fraction of monomers in the folded conformation increased with a decrease in the concentration of NaCl below 0.2 M, and the conformational state was affected neither by the presence of ATP nor by the phosphorylation of regulatory light chain. In most of the folded monomers, the tail bent back toward the heads at one region, 45 nm apart from the head-tail junction, and the remaining tail portion containing the C-terminal tip appeared to interact with the head-tail junction. Only a small percentage of the folded monomers was in a more compact conformation close to the 10 S conformation of vertebrate smooth muscle and non-muscle myosins. The folded monomers, however, may not trap the products of ATP hydrolysis as assessed by single turnover experiments. The percentage of monomers in the 10 S-like conformation was increased by the exchange of a regulatory light chain with the smooth muscle light chain, indicating the participation of head-tail junction, including the regulatory light chain in the formation of folded conformation. The folded conformation may be common to various myosin IIs, suggestive of common roles for the folded monomers.

Myosin II consists of two globular heads connected to one long α-helical coiled coil tail. Each head contains an ATPase site, an essential light chain, and a regulatory light chain (RLC).1 Under physiological ionic conditions in the presence of ATP, vertebrate smooth muscle and non-muscle myosins are folded into the 10 S folded conformation in which the tail bends at two regions (1–3) and are unfolded to the 6 S extended conformation. The tail bends at one region, 45 nm apart from the head-tail junction, and the remaining tail portion containing the C-terminal tip appeared to interact with the head-tail junction. Only a small percentage of the folded monomers was in a more compact conformation close to the 10 S conformation of vertebrate smooth muscle and non-muscle myosins. The folded monomers, however, may not trap the products of ATP hydrolysis as assessed by single turnover experiments. The percentage of monomers in the 10 S-like conformation was increased by the exchange of a regulatory light chain with the smooth muscle light chain, indicating the participation of head-tail junction, including the regulatory light chain in the formation of folded conformation. The folded conformation may be common to various myosin IIs, suggestive of common roles for the folded monomers.

MATERIALS AND METHODS

Proteins—Skeletal muscle myosin was prepared from rabbit back muscle as described by Perry (12), and smooth muscle myosin was from porcine aorta as described previously (13, 14). Nonphosphorylated and phosphorylated aorta RLC were prepared as described previously (15). Rabbit skeletal muscle myosin light chain kinase was prepared according to the method of Nagamoto and Yagi (16). Skeletal myosin was phosphorylated under the same conditions as those for the phosphorylation of smooth muscle myosin (17), except for the use of skeletal myosin light chain kinase. The phosphorylated myosin was purified in two cycles of precipitation by dilution with a low salt solution and by dissolving it in a high salt solution. The RLC of the obtained preparation was completely phosphorylated as judged by urea-gel electrophoresis (18). Rabbit skeletal myosin in which RLC was exchanged to aorta RLC was prepared by incubating myosin at 37 °C with an 8-fold molar excess of aorta RLC as described previously (19). The RLC in skeletal myosin was completely exchanged to aorta RLC, as judged by urea-gel electrophoresis (18).

Electron Microscopy—Myosin (0.2 mg/ml) in 0.12–0.5 M NaCl, 1 mM MgCl₂, 0.1 mM EGTA, and 10 mM imidazole (pH 7.0) was ultracentrifuged at 150,000 × g for 10 min at 4 °C in the presence or absence of 1 mM ATP. The myosin remaining in the supernatant was cross-linked with 5 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) for 30 min at 25 °C. After the reaction was terminated with ~5 mM dithiothreitol, the cross-linked sample was diluted 3–20-fold with 70% glycerol and 0.4 M ammonium acetate (pH 7.2) or with 70% glycerol, 0.1 mM MgATP, 0.1 mM EGTA, and 50 mM ammonium acetate (pH 7.2). The diluted sample sprayed onto mica was rotary shadowed with platinum and observed with a Hitachi H-800 electron microscope (20).

Gel Filtration HPLC—Myosin (0.2 mg/ml) in a solution containing 0.08–0.4 M NaCl, 1 mM MgCl₂, 0.1 mM EGTA, and 10 mM imidazole (pH 7.0) in the presence or absence of 0.1 mM ATP was passed through a 0.5-μm membrane filter (Millipore) to remove filaments. The myosin was then analyzed by gel filtration HPLC at the same NaCl concentration as above with 1 mM MgCl₂, 0.1 mM EGTA, and 10 mM sodium phosphate (pH 7.2) in the presence or absence of 20 μM ATP, respectively, at room temperature, using a TSK gel G5000PWXL column (7.8 × 300 mm) with a TSK guard column PWXL (6.0 × 40 mm) (20).

Measurement of the Rate of Product Release from Myosin—[γ-32P]ATP (0.5 μM) was mixed with nonphosphorylated myosin (0.02 mg/ml, 0.05 μM head) in 0.12–0.4 M NaCl, 1 mM MgCl₂, 0.1 mM EGTA, 1 mM dithiothreitol, and 20 mM Tris-HCl (pH 7.5). After the mixture was incubated for 2 min at 25 °C, 1 μM unlabeled ATP was added. Because of the limited concentration of monomeric myosin, the incubation was prolonged to 2 min to maximize the amount of [γ-32P]ATP bound to myosin. At various times, a 150-μl aliquot of the reaction mixture was filtered with a Millipore Ultrafree-MC centrifugal filter unit (100,000 nominal molecular weight limit) at 5,000 × g for 1 min at 25 °C. Aliquots (50 μl) of the filtrate and reaction mixture without filtration were counted in a liquid scintillation counter (Beckman LS6000SE). The amount of phosphate bound to myosin was estimated by subtracting the amount of phosphate in the filtrate from that in the reaction mixture.

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2 To whom correspondence should be addressed: Tel.: 81-11-706-3814; Fax: 81-11-706-4924.
3 The abbreviations used are: RLC, regulatory light chain; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; HPLC, high performance liquid chromatography.
Monomeric Conformation of Skeletal Muscle Myosin

RESULTS AND DISCUSSION

Smooth muscle and non-muscle myosin filaments disassemble to 10 S folded monomers after the addition of MgATP under physiological ionic conditions (~0.15 M NaCl, pH 7.0) when they are not phosphorylated (3–6), whereas rabbit skeletal muscle myosin filaments do not. Under these conditions, a small amount of skeletal muscle myosin (~0.02 mg/ml) remains monomeric. After filamentous myosin was pelleted by ultracentrifugation, the conformation of myosin monomers remaining in the supernatant was fixed by cross-linking with EDC. The cross-linked sample was diluted with a solution containing 70% glycerol and 0.4 mM ammonium acetate (pH 7.2), rotary shadowed, and examined by electron microscopy. Many of the myosin monomers were observed to have a folded structure in which the tail bent back toward the heads at one region, and the tail portion containing the C-terminal tip appeared to interact with the head-tail junction (referred to as an open folded conformation) (Figs. 1a and 2a). When the salt concentration was increased to 0.4 M prior to the cross-linking, most of the monomers were in the extended conformation (Fig. 1b). The salt concentration-dependent conformational change was observed, irrespective of the presence of ATP. The number of folded monomers was 62% at 0.12 M NaCl in the presence of ATP but was only 7% at 0.4 M NaCl. It was 57% at 0.13 M NaCl in the absence of ATP but was only 7% at 0.4 M NaCl (Table I).

Most of the folded monomers of skeletal myosin, however, were in the open conformation, with only a low percentage in the 10 S myosin-like conformation in which the tail bends at two regions (referred to as a closed folded conformation). When the cross-linked sample was diluted with a glycerol solution more favorable for the folded conformation (50 mM ammonium acetate), the percentage of folded monomers in the closed conformation increased significantly, but the majority of folded monomers were still in the open conformation, with only a low percentage in the closed conformation (Table I).

The bending position measured for the skeletal folded monomers was 65 ± 5 nm (n = 54) apart from the head-tail junction as normalized with a tail length of 155 nm (21). This value is in agreement with the reported value for the first hinge in the skeletal muscle myosin tail (21), and is considerably shorter than the corresponding length in gizzard 10 S myosin (51 nm) (1). Skeletal myosin monomers in the closed conformation were similar in appearance to smooth muscle 10 S myosin, but the size of the folded tail was different (Fig. 2, b and d). The length from the tip of tail to the second binding region that appeared to interact with the region at or near the head-tail junction was determined to be 76 ± 6 nm (n = 23), which is significantly longer than the corresponding value (60

![Fig. 1. Rotary-shadowed rabbit skeletal muscle myosin molecules.](image)

Nonphosphorylated myosin monomers in the presence of 1 mM ATP were rotary-shadowed after cross-linking with EDC. a, rabbit skeletal myosin at 0.15 M NaCl; b, rabbit skeletal myosin at 0.4 M NaCl to which the salt concentration of a monomer solution obtained at 0.13 M NaCl was increased prior to the cross-linking; c, RLC-exchanged skeletal myosin containing RLC at 0.15 M NaCl. Arrowheads indicate myosin molecules in the open folded conformation (b) and in the closed folded conformation (c). Scale bar, 100 nm.

<table>
<thead>
<tr>
<th>Myosin</th>
<th>NaCl</th>
<th>ATP</th>
<th>n</th>
<th>Folded (open:closed)</th>
<th>Extended</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit skeletal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>0.13</td>
<td>138</td>
<td>57</td>
<td>94:6</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>141</td>
<td>62</td>
<td>98:2</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>103</td>
<td>56</td>
<td>98:2</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>0.15*</td>
<td>100</td>
<td>94</td>
<td>82:12</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>0.40*</td>
<td>182</td>
<td>7</td>
<td>85:15</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>312</td>
<td>7</td>
<td>90:10</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>195</td>
<td>7</td>
<td>92:7</td>
<td>93</td>
</tr>
<tr>
<td>RLC-exchanged</td>
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<td>469</td>
<td>77</td>
<td>69:32</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>199</td>
<td>11</td>
<td>95:5</td>
<td>89</td>
</tr>
<tr>
<td>Porcine aorta</td>
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<td>164</td>
<td>84</td>
<td>79:3</td>
<td>16</td>
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<tr>
<td>Native</td>
<td>0.30</td>
<td>110</td>
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<td>22:78</td>
<td>39</td>
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<tr>
<td></td>
<td>0.40</td>
<td>121</td>
<td>3</td>
<td>0:100</td>
<td>97</td>
</tr>
</tbody>
</table>

*The supernatant obtained at 0.15 M NaCl was diluted 10-fold with 70% glycerol, 0.1 mM MgATP, 0.1 mM EGTA, and 50 mM ammonium acetate (pH 7.2) instead of the above solution.

The salt concentration of the supernatants obtained at 0.13 M NaCl in the absence and presence of ATP, respectively, was increased to 0.4 M prior to the cross-linking.
nm) in gizzard 10 S myosin (1).

The difference of the major conformations between the folded monomers of skeletal and smooth myosins might be due to the difference in their RLCs. In fact, Trybus and Lowey (22) reported that smooth muscle myosin in which RLC was replaced with skeletal RLC forms a partially folded conformation that is similar to the open folded conformation of skeletal myosin (Figs. 1 and 2). To test this possibility, the conformation of skeletal myosin in which RLC was replaced with aorta RLC was examined. The replacement with the smooth muscle RLC markedly increased the percentage of folded monomers in the closed conformation (Fig. 1c and Table I) but essentially did not affect the amount of monomers formed, irrespective of the phosphorylation state (data not shown). The structure of the closed conformation of the RLC-exchanged skeletal myosin was indistinguishable from that of unexchanged skeletal myosin (Fig. 2b and c). Smooth muscle RLC may stabilize the closed folded conformation more than skeletal RLC. The region of the head-tail junction including RLC may have an important role in forming the folded structure in skeletal myosin as suggested for the folding of smooth muscle myosin (17, 22, 23).

The conformational transition of skeletal myosin monomers was also examined by gel filtration HPLC on which the 6 S extended and 10 S folded monomers of smooth muscle myosin have been shown to be eluted at different times (20, 22) (Fig. 3a). The elution time for skeletal myosin increased gradually as the concentration of NaCl decreased to below 0.2 M, irrespective of the presence of ATP, consistent with the results observed upon electron microscopy (Table I and Figs. 1 and 2). The gradual change in elution time suggests rapid equilibrium between the extended and folded forms and a salt concentration-dependent shift of equilibrium. The elution profile, however, was independent of the phosphorylation of RLC and of the presence of ATP (Fig. 3b). Replacement with aorta RLC did not change the ATP and phosphorylation independencies but slightly increased the elution time (Fig. 3b), consistent with the results of electron microscopy (Table I).

As one of the profound features, the folded monomers of myosins from vertebrate smooth muscle and non-muscle and from scallop striated muscle are shown to trap the products of ATP hydrolysis, ADP and Pi, in the active site (8, 10, 11). To examine whether the folded monomers of skeletal myosin trap the hydrolysis products, single turnover experiments were carried out with 0.02 mg/ml nonphosphorylated myosin. The rate of phosphate release from aorta nonphosphorylated myosin at 0.12 M was \(-0.0004\) s\(^{-1}\), and the amount of phosphate bound to myosin at 0 time was estimated to be 0.7 mol/mol head, consistent with the previous results (10, 11). The rate was increased to \(-0.004\) s\(^{-1}\) with an increase in the concentration of NaCl to 0.3 M at which the fraction of folded monomers in aorta myosin was comparable with that in skeletal myosin at 0.12 M NaCl (Table I). However, the rate for skeletal myosin at 0.12 M NaCl was higher than 0.015 s\(^{-1}\), which was significantly higher than the value for aorta myosin at 0.3 M NaCl and was similar to the values for aorta and skeletal myosins at 0.4 M NaCl at which these myosins were both in the extended conformation (Table I and Fig. 3). These results indicate that skeletal myosin monomers in the open folded conformation may not trap the products of ATP hydrolysis.

Potential formation of a folded structure in skeletal myosin
has been shown by negative staining of myosin in a high salt solution (21). Here we showed that skeletal myosin was folded under physiological conditions as observed for smooth muscle and non-muscle myosins, suggestive of common roles for the folded monomers in these myosins. However, such folded monomers have been detected only in gizzard smooth muscle cells (24), and the actual biological significance of folding remains unknown. Of potential significance common to the folded monomers is their compact form, which is suitable for transport in cells. This seems to be particularly important in non-muscle cells in which myosin molecules must be transported to the intracellular locus where contractile activity is needed. In muscle cells, the transportation of myosin molecules may also be required for the formation of thick filaments at appropriate locations, at least at certain stages of development and/or for the turnover of myosin molecules in the thick filaments. In line with this speculation, the existence of folded monomers is also mentioned for vertebrate cardiac myosins (8). In addition to vertebrate myosins, molluscan smooth and striated muscle myosins have been shown to form the folded conformation (7–9).

The conformational transition of myosins other than vertebrate striated myosin can be regulated by phosphorylation of RLC or the tail, or by the binding of Ca$^{2+}$ (2–5, 7–9). The folded monomers of these myosins are stable and thus resistant to filament assembly until regulatory signals promote unfolding. The folded monomers trap the products of ATP hydrolysis, thus their ATPase activities are very low (8, 10, 11). Such properties would be suitable for cells in which relatively large amounts of myosin molecules need to be transported. On the other hand, the conformational transition of skeletal myosin monomers is not regulated, and folded monomers are unstable under physiological conditions and may not trap the products of ATP hydrolysis. Only a small amount of myosin may need to be transported in skeletal muscle cells. The transported folded monomers are in rapid equilibrium with the extended monomers, which can easily be incorporated or exchanged into myosin filaments. Therefore, the myosin filaments are stabilized without a distinct regulatory mechanism and are ready for rapid contraction in skeletal muscle cells.

REFERENCES

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